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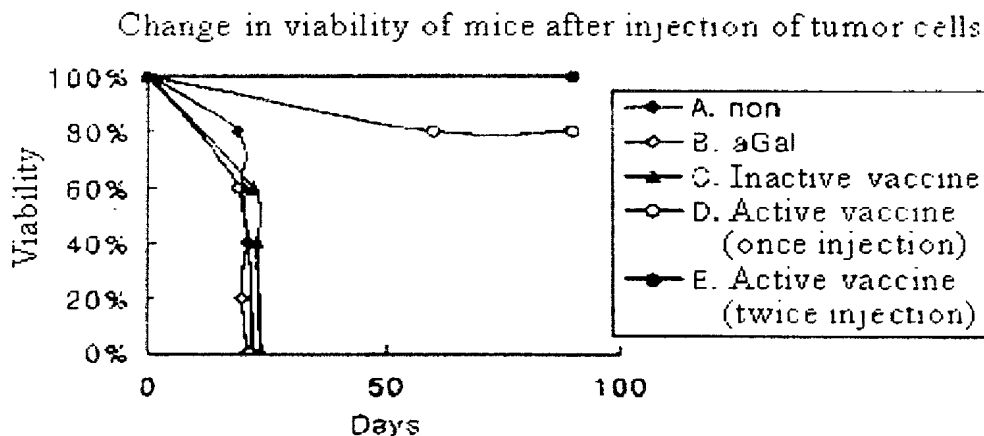
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(54) Title: PHARMACEUTICAL INGREDIENT FOR MEDICAL TREATMENT AND PREVENTING OF CANCER



(57) Abstract: Pharmaceutic ingredient for medical treatment and prevention of cancer in this invention is consist of tumor cell extract as antigens and materials which can stimulate NKT cells at the time of antigen presentation to boost co-stimulatory molecule expression in the antigen presenting cells. The active vaccine successfully protected mice received live tumor cells two weeks after vaccine immunization. It also could enhance the survival of mice, which received live tumor cells before vaccine immunization. The effect of cancer vaccine is absolutely dependent of NKT cells since any vaccine mediated protective effect was not observed in CD1d deficient mice, which do not have NKT cells. Enhanced cytotoxic effect by the conventional CD4+ or CD8+ memory T cells seem major contributor for the rejection of newly injected or already established tumors. NKT cells do not directly involved in the cytotoxicity to kill the tumor cells but enhance the development tumor specific memory T cells.



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# PHARMACEUTIC INGREDIENT FOR MEDICAL TREATMENT AND PREVENTION OF CANCER

## TECHNICAL FIELD

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The present invention is generally related to pharmaceutical ingredients for treatment and prevention of cancer, and more particularly, a pharmaceutical preparation for treating and preventing cancer, comprising killed tumor cells and a material capable of inducing production of co-stimulatory molecules in antigen presenting cells and thus stimulating natural killer T cells.

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## PRIOR ART

Cancer is still a representative disease threatening the health and lives of humans, especially in industrialized countries, and as such is a major cause of death in humans. The exact  
15 cause of cancer remains unclear, but cancer is generally attributed to diverse factors, including internal factors such as genetic elements as well as external factors such as carcinogens, radiation, ultra violet, cosmic ray, chronic inflammation and injuries lasting for a long period of time, and oncogenic viruses. The potential involvement of genetic factors in cancer development is demonstrated in experimental animal models. However, there is insufficient knowledge of the  
20 role of genetic factors in induction of human cancer, except some cancers including retinoblastoma and familial adenomatous polyposis. Furthermore, it is not clear whether human cancer is caused by the genetic factors or environmental factors.

Cancer is not absolutely incurable, and moreover, can be fully overcome with aggressive treatment, especially when being found at an early stage. Therefore, it is important to detect  
25 cancer at an early stage, thus enhancing effectiveness of medical treatments. Also, even in case of

progressed cancer, if diverse and aggressive treatments are used, the fatal disease can be conquered, or patients' lives can be prolonged with reduced the pain. Further, it is most preferable that cancer progress is prevented. Therefore, there is an urgent need for development of therapeutic agents, such as vaccines to treat or prevent cancer.

5 Cancer vaccines for effective prevention and treatment of cancer are generally developed based on immunotherapy.

Immune response is initiated by recognition of antigen by T cells. T cells recognize an antigen presented by antigen presenting cells (APC) such as dendritic cells or macrophages. In detail, after being processed, the antigen forms a complex with antigen presenting molecules such as major histocompatibility complex (MHC) on APC, and T cells recognize the antigen-MHC molecule complex through the antigen-specific receptor, which exists on T cells, thus activating T cells and consequently triggering immune response.

However, the immune system recognizes cancer cells as "self". Therefore, to obtain protective effects of cancer vaccines in cancer immunotherapy, priority should be given to recognition of cancer cells as "non-self".

To achieve this goal, immunological tolerance/ignorance against self antigens presented by cancer cells should be overcome. Even though B cells or T cells recognizing specific antigens on cancer cells exist, they are typically killed, inhibited or inactivated as a result of immunological tolerance. Taking a detailed look at T cells, T cells are fully activated when recognizing antigens through T cell Receptor (TCR), as well as the co-stimulatory molecule, B7-1 or B7-2 molecule expressed on the surface of antigen presenting cells such as dendritic cells (DC) or macrophages, which act as a co-stimulatory signal. Upon recognizing only tumor-derived antigens with no recognition of the co-stimulating molecules, T cells are typically deleted, inhibited or inactivated. When cancer develops, typically, only tumor-specific antigens from tumor cells are produced, while the co-stimulatory molecules are not expressed, and immune cells capable of recognizing

and attacking the tumor cells are thus continuously removed or inactivated, resulting in the proliferation of the tumor cells with no of interruption of immune system. Therefore, in therapy and prevention of tumor, it is of essential importance for the immune system to recognize tumor-specific antigens simultaneously with recognition of the co-stimulatory molecules.

5

#### DISCLOSURE OF THE INVENTION

It is therefore an object of the present invention to provide a pharmaceutical preparation for treating and preventing cancer, which can induce immune response against tumor cells by recognizing  
10 tumor cells as tumor-specific antigens, not “self”, and augmenting expression of a co-stimulating molecule required for maximal T cell activation.

It is another object of the present invention to provide a pharmaceutical preparation for treating and preventing cancer using natural killer T cells (NKT cells) in order to increase expression of a co-stimulatory molecule to induce activation of T cells.

15 To achieve the objects described above, in accordance with an aspect of the present invention, there is provided a pharmaceutical preparation for treating and preventing cancer, comprising a biologically effective amount of killed tumor cells, which act as antigens, and a biologically effective amount of a material to increase expression of a co-stimulatory molecule required for maximal T cell activation as an effective ingredient.

20 In accordance with the present invention, the killed tumor cells are characterized by being dead by undergoing necrosis.

In accordance with the present invention, the co-stimulatory molecule is a B7-1 or B7-2 molecule.

In accordance with the present invention, the material to increase expression of the B7-1 or B7-2 molecule as the co-stimulating molecule is characterized by having ability to stimulate NKT cells,  
25 which activate antigen presenting cells (APC) expressing the B7-1 or B7-2 molecule.

In accordance with the present invention, the material to activate NKT cells is glycolipid or lipid acting as a ligand for CD1d on APC that present antigen to NKT cells.

In accordance with the present invention, the glycolipid is alpha-galactosylceramide (alpha-GalCer).

- 5 In accordance with the present invention, the material to activate NKT cells is an antibody selectively recognizing and activating NKT cells.

In addition, in accordance with the present invention, the material to activate NKT cells is characterized by having ability to stimulate T-cell receptor (TCR) on NKT cells and thus activate NKT cells.

10

#### BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the  
15 accompanying drawings, in which:

FIG. 1 is a graph showing the effect of cancer vaccine prepared in Experimental Example 1 on viability of cancer-induced mice;

FIG. 2 is a graph showing the effect of cancer vaccine over a prolonged period of time;

FIG. 3 shows an expression pattern of B7.2 molecule after stimulation of NKT cells by alpha-  
20 GalCer injection in mice;

FIG. 4 is a graph showing pretreatment effect of alpha-GalCer at various times;

FIG. 5 is a graph in which viability of each group of Experimental Example 4 is plotted against time (days);

FIG. 6 is a graph in which viability of each group of Experimental Example 5 is plotted against  
25 time;

FIG. 7 is a photograph showing change in frequency of NKT cells in mouse liver at 16 hours after being treated with alpha-GalCer, which is one ingredient of an active vaccine of Experimental Example 6;

FIG. 8 is a graph showing a result of a cytotoxicity test of spleen cells (effector cells) against male mice-derived cells (target cells), which are derived from female mice immunized with spleen cells from male mice, along with treatment with alpha-GalCer; and

FIG. 9 is a graph showing a result of a cytotoxicity test of spleen cells (effector cells) against live tumor cells (target cells), which are derived from female mice immunized with an active vaccine or an inactive vaccine according to the present invention.

10

#### BEST MODES FOR CARRYING OUT THE INVENTION

In accordance with an embodiment of the present invention, necrotic tumor cells function as antigens, alpha-GalCer, which is a potent activating ligand presented by CD1d acting as an antigen presenting molecule on APC that presents antigen NKT cells, is employed to increase production of a co-stimulatory molecule required for maximal T cell activation.

The CD1d molecule, which is a sort of antigen presenting molecule, belongs to group 2 of the CD1d family, and exists in both humans and mice. Mouse CD1d molecule is mainly expressed in APC such as dendritic cells, B cells or macrophages (Brossay, L., et al. Mouse CD1 is mainly expressed on hemopoietic-derived cells. J. Immunol. 159:1216-1224 (1997); and Jessica H. Roark, Se-Ho Park, Jayanthi Jayawardena, Uma Kavita, Michele Shannon, and Albert Bendelac. CD1.1 expression by mouse antigen presenting cells and marginal zone B cells. J. Immunol. 160:3121-3127 (1998)).

Natural killer T cells (NKT cells), which is a novel T-cell lineage selected by the CD1d molecule, especially in mouse, semi-permanently express T cell Receptor (TCR), are activated by CD1d,

and express cell surface antigens similar to those on natural killer cells (NK cells). NKT cells are highly conserved in humans as well as mice, and represent 1-30 % of total T cells depending on tissues.

Lipid or glycolipid is predominantly considered to be a ligand for the CD1d molecule. It is reported that glycolipid phosphatidylinositol associates with CD1d (Joyce, S., et al. Natural ligand of mouse CD1d1: cellular glycosylphosphatidylinositol. *Science* 279:1541-1544 (1998); and Gumperz, J. E., et al. Murine CD1d-restricted T cell recognition of cellular lipids. *Immunity* 12:211-221 (2000)), and glycolipid isolated from poriferans, alpha-galactosylceramide (alpha-GalCer) also associates with CD1d (Kawano, T., et al. CD1d-restricted and TCR mediated activation of V $\alpha$ 14 NKT cells by glycosylceramides. *Science* 278:1626-1629 (1997); Benlagha, K., et al. In vivo identification of glycolipid antigen specific T cells using fluorescent CD1d tetramers. *J. Exp. Med.* 191:1895-1903 (2000); and Spada, F.M., Koezuka, Y., and Porcelli, S. A. CD1d-restricted recognition of synthetic glycolipid antigens by human natural killer T cells. *J. Exp. Med.* 188:1529-1534 (1998)). Alpha-GalCer stimulates V $\alpha$ 14 TCR even in an infinitesimal amount in both humans and mice by associating with CD1d.

When the pharmaceutical preparation for treating and preventing cancer according to an embodiment of the present invention is administered to mice or humans, APC process necrotic tumor cells, and alpha-GalCer is presented by CD1d to NKT cells, thus activating NKT cells. The activated NKT cells secrete a variety of cytokines including IL-4 and IFN-gamma (Bendelac, Albert, Miguel N. Rivera, Se-Ho Park, and Jessica H. Roark. Mouse CD1-specific NK1 T cells. Development, Specificity, and Function. *Annu. Rev. Immunol.* Vol. 15:535-562 (1997); and Yoshimoto, T., and Paul, W. E. CD4<sup>+</sup>NK1.1(+) T cells promptly produce IL-4 in response to in vivo challenge with anti-CD3. *J. Exp. Med.* 179:1285 (1994)), and these cytokines activate professional APCs such as macrophages or dendritic cells (DC), which present tumor antigens, as well as increase expression of the co-stimulatory molecules including B7-1 and B7-2



molecules, required for maximal T cell activation. T cells recognize antigens presented by CD1d as well as the co-stimulatory molecule such as B7-2 through a receptor for CD1d and CD 28 receptor for the co-stimulatory molecules. As described above, when the immune cells recognize tumor-specific antigens presented on APCs, the co-stimulatory molecules help to  
5 overcome immunological tolerance induced by tumors, with no deletion or inactivation of T cells, resulting in activation of T cells to memory T cells.

In addition, because of stimulating secretion of cytokines such as IL-4, IFN-gamma or IL-12, simultaneously with a series of activation of NKT cells, dendritic cells, B cells and T cells, the pharmaceutical preparation according to the present invention may be effective for therapy and  
10 prevention of infectious diseases and autoimmune diseases as well as cancer.

The pharmaceutical preparation according to the present invention can be used for diagnosis and therapy of cancer, as well as being utilized as a cancer vaccine with the aim of preventing cancer.

In addition, the pharmaceutical preparation for treating and preventing cancer according to the present invention can be formulated into a solid, semi-solid or liquid form pharmaceutically  
15 suitable for oral or parenteral administration, in combination with a pharmaceutically acceptable excipient, thus allowing oral or parenteral administration. The pharmaceutically acceptable excipient may be solid or liquid, and includes one or more selected from materials capable of augmenting or regulating immune response.

A unit dosage of the pharmaceutical preparation for treating and preventing cancer according to  
20 the present invention may vary depending on a patient's age and pathogenic state or pharmaceutical formulation thereof. The preferred unit dosage of the pharmaceutical preparation can be easily determined by those skilled in the art.

Necrotic tumor cells contained in the pharmaceutical preparation according to the present invention may vary depending on kinds of cancer or therapeutic purposes, and can be selected  
25 based on clinical data including kinds of cancer and developed states of cancer.

The present invention will be explained in more detail with reference to the following examples in conjunction with the accompanying drawings. However, the following examples are provided only to illustrate the present invention, and the present invention is not limited to them.

## 5 EXAMPLE

### EXPERIMENTAL EXAMPLE 1

An effectiveness of a pharmaceutical preparation for treating and preventing cancer according to  
10 the present invention was evaluated in mice, as follows.

#### 1. Preparation Step

An effect of the pharmaceutical preparation was investigated using C57BL/6 mice and a mouse melanoma cell line, B16, as well as alpha-galactoceramide (alpha-GalCer), KRN7000, to selectively activate NKT cells.

15 To increase antigenicity of B16 melanoma used as tumor-derived antigens, the tumor cells were killed by necrosis, thus allowing their recognition by the immune system as if self tumor cells had been killed owing to invasion of pathogenens or injury. Necrotic cells more strongly stimulate the immune system than those killed by apoptosis (Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. Nature Medicine 5(11):1249-55 (1999)).  
20 When employing apoptotic cells, lower efficacy was found in comparison with using necrotic cells, which will be described in more detail in the below Examples.

When stimulating NKT cells, APCs such as macrophages or dendritic cells can be activated and expression of the co-stimulatory B7-2 molecule can be thus increased, thereby enabling T cells to be activated by recognizing tumor-specific antigens presented by APCs and then recognizing and  
25 destroying tumor cells.

## 2. Preparation and use of a pharmaceutical preparation for treating and preventing cancer

B16 melanoma cells (obtained from Dr. Rosenberg's lab, NIH) were cultured in 10 % FBS-containing RPMI medium, and washed several times with PBS to completely remove medium.

5 B16 cells were then prepared in  $1 \times 10^7$  cells/ml in PBS, and necrosis was induced by repeating four freezing ( $-70^\circ\text{C}$ ) and thawing ( $37^\circ\text{C}$ ) steps. Microscopic investigation showed no presence of cells having normal morphology. When being cultured again for over 2 weeks, no surviving cells were observed. To prepare a vaccine, the necrotic cancer cells were mixed with alpha-GalCer of  $20\text{ }\mu\text{g/ml}$  in PBS (Kirin Beer Co., Ltd., Japan) in a volume ratio of 1:1 to give a  
10 vaccine comprising  $5 \times 10^7$  cells/ml of cancer cells and  $10\text{ }\mu\text{g/ml}$  of alpha-GalCer, which was stored at  $-70^\circ\text{C}$  until use (hereinafter, the vaccine is referred to as "active vaccine" or "active cancer vaccine"). As a control, another vaccine (hereinafter, referred to as "inactive vaccine" or "inactive cancer vaccine") was prepared by mixing the same amount of the necrotic tumor cells with PBS in a volume ratio of 1:1, and a solution composed of alpha-GalCer of  $10\text{ }\mu\text{g/ml}$   
15 (hereinafter, referred to as "alpha-GalCer"), not containing the necrotic tumor cells, was prepared.

## 3. Maintenance and immunization of mice

In order to evaluate effects of the active vaccine, 6-8 weeks aged C57BL/6 mice were immunized. All experiments were performed under the same environment using the same aged  
20 mice. To reduce experimental errors, mice of each group were maintained in the same cage, not isolated from each other. That is, one mouse of each of groups A to E was maintained in one cage, and about 10 such cages were further prepared, thereby minimizing potential errors in a specific cage.

2 weeks before injection of live tumor cells, mice were immunized. Group A was not  
25 immunized. Groups B, C and D were immunized by intraperitoneally injecting 0.1 ml of alpha-

GalCer, inactive vaccine and active vaccine, respectively. Group E was twice immunized by intraperitoneally injecting active vaccine 2 and 4 weeks before injection of live tumor cells.

Thereafter, live tumor cells were injected intraperitoneally to mice of groups A to E, and growth rate of cancer and viability of mice were observed, in which the injection point was set as day 0.

- 5 Mice having a distended abdomen owing to proliferation of cancer cells and remarkably reduced capacity of locomotion died a natural death, and mice obviously expected to die were killed by cervical dislocation.

#### 4. Result

- 10 FIG. 1 shows an effect of cancer vaccine on viability of cancer-induced mice. As shown in FIG. 1, after about 3 weeks, all mice of groups A to C were found to be dead. In contrast, when being administered with active vaccine, all mice except one mouse survived, while maintaining a healthy state, in which tumor cells were not found in their abdomen. Also, between groups D and E administered with the active vaccine once and twice, respectively, only a negligible difference was
- 15 found. These results indicate that treatment with only tumor cells or alpha-GalCer has no effectiveness in therapy or prevention of cancer. Moreover, when repeatedly performing the experiment, mice treated with alpha-GalCer showed lower viability. However, when being injected with both the necrotic tumor cells and alpha-GalCer, the mice amazingly showed about 100 % viability, indicating that the immune system recognized the necrotic tumor cells as non-self
- 20 antigens and thus induced immune response, in which alpha-GalCer plays an critical role, and the action of alpha-GalCer is accomplished in the presence of tumor antigens.

#### EXPERIMENTAL EXAMPLE 2

- 25 The long-term effect of the active vaccine as a candidate cancer vaccine was evaluated as follows.

### 1. Treatment with the active vaccine and injection with tumor cells

First, mice were treated with the active vaccine. After about 6 months, mice were injected with live tumor cells, in which mice were divided into group "non" not treated with any vaccine, group "d-150" treated with the active vaccine 5 months before tumor cell injection, and group "d-14" treated with the active vaccine 2 weeks before tumor cell injection. All mice were maintained under same environment. The injection point of the active vaccine was set as day 0, and growth rate of tumor cells and viability of mice were evaluated according to the same method as in Experimental Example 1.

### 2. Result

FIG. 2 shows the long-term effect of the active vaccine. As shown in FIG. 2, it was found that proliferation of tumor cells is inhibited and tumor cells finally died in group "d-14" and group "d-150" treated with the active vaccine 2 weeks and 5 months before tumor cell injection, respectively, but not in the group "non". These results indicate that the active vaccine according to the present invention is a potential cancer vaccine with long-term preventive effects, even with only one injection.

The efficacy as a cancer vaccine of the active vaccine, described in the Experimental Examples 1 and 2, originates from the result that NKT cells activated by alpha-GalCer give a signal to activate the immune cells, not to induce immunological tolerance, when DCs present killed tumor cells to the immune cells. This fact is supported by the finding in that alpha-GalCer does not activate DCs in NKT cell-deleted mice, while activating DCs in normal mice having NKT cells.

FIG. 3 shows the presence of NKT cells and expression pattern of the co-stimulatory B7-2 molecule. After injecting alpha-GalCer to normal mice having NKT cells and CD1<sup>-/-</sup> mice

with deletion of NKT cells, activation of DCs was evaluated by investigating expression levels of B7-2 molecule. As shown in FIG. 3, B7-2 expression was increased in normal mice by injection of alpha-GalCer, whereas being unchanged in CD1<sup>-/-</sup> mice. This result indicates that the immune response against the necrotic tumor cells occurs by a mechanism in which alpha-GalCer activates NKT cells, and the activated NKT cells then activate DCs, thus leading to increased production of B7-2 molecule in DCs.

### EXPERIMENTAL EXAMPLE 3

The following experiment was performed to identify whether alpha-GalCer shows antitumor immunity by a mechanism different with that of the necrotic tumor cells contained in the active vaccine.

#### 1. Pretreatment of alpha-GalCer at various times

After being pretreated with alpha-GalCer (2 µg/mouse, i. v.) at various times, mice were injected with tumor cells, and the growth level of tumor cells was then investigated. 1×10<sup>6</sup> tumor cells were suspended in 100 µl PBS to facilitate their transition to the liver and injected to the spleen using 30 gage syringes. Immediately after injection of tumor cells, a vein extended to the liver was tied up with a surgical silk thread (6-0 silk, Sherwood Medical, St. Louis, MO), and this surgical operation was carried out within about 5 min after anesthetizing mice with avertin (300 µl of 2.5 % Avertin/20g body weight, i. p.). 24 hours after the injection of tumor cells (day 1), mice were treated again with alpha-GalCer (2 µg/mouse, i. p.) to activate NKT cells and thus destroy tumor cells. 10 days after the injection of tumor cells (day 10), the liver was excised from each mouse, and transition of tumor cells was evaluated and liver weight was measured.

## 2. Result

FIG. 4 shows pretreatment effect of alpha-GalCer at various times, in which times at which pretreatment of alpha-GalCer was performed are given on the x-axis (for example, -2 hr means that alpha-GalCer was pretreated 2 hours before the injection of tumor cells, and -5 d means pretreatment 5 days before the injection of tumor cells), and weight of liver (g/liver) is given on the y-axis and increased according to transition and proliferation of tumor cells. As shown FIG. 4, in the group pretreated with PBS and then injected with tumor cells (rightmost), not treated with alpha-GalCer, the liver was found to be heaviest among all groups, indicating that proliferation of tumor cells is not inhibited. In the group pretreated with alpha-GalCer, injected with tumor cells and then not treated with alpha-GalCer, weight of liver was found to have reduced. When being pretreated with PBS or alpha-GalCer 2 hours before the injection of tumor cells and treated with alpha-GalCer, mice showed reduced liver weight. In the groups pretreated with alpha-GalCer once 5 days before, or twice, 10 days and 5 days before the injection of tumor cells, and treated with alpha-GalCer, weight of liver was not found to decline.

In other words, in the groups pretreated with alpha-GalCer once 5 days before, or twice, 5 and 10 days before the injection of tumor cells, and treated with alpha-GalCer, proliferation of tumor cells was not controlled effectively, in comparison with the groups pretreated with alpha-GalCer 2 days before or not, and moreover, when pretreatment was performed a longer time before, the control of tumor cells was more difficult. These results indicate that treatment with alpha-GalCer leads to increase in death rate of NKT cells, which is higher with the lapse of time, and reduction in ability of NKT cells to directly attack tumor cells.

When being treated with alpha-GalCer, APCs are activated and then activate NKT cells, and the NKT cells secrete various cytokines. Some cytokines activate natural killer cells (NK cells) to produce killer molecules such as cytokine perforin, other cytokines directly attack tumor cells.

When being treated with alpha-GalCer once, regardless of being pretreated or treated after the

injection of tumor cells, the resulting powerful antitumor activity originates from the temporal effect of the action of alpha-GalCer. However, NKT cells activated by alpha-GalCer were killed, resulting in the reduced ability to directly destroy tumor cells. Therefore, these results demonstrate that alpha-GalCer alone has no efficacy as a cancer vaccine.

5 Also, these results indicate that the temporal antitumor activity of alpha-GalCer is mediated by a mechanism completely different from the immune response induced by the necrotic tumor cells contained in the cancer vaccine according to the present invention, and that the activated NKT cells play a critical role in increasing titer of the necrotic tumor cells as antigens, and any material capable of activating NKT cells, such as alpha-GalCer, is useful in the cancer vaccine according  
10 to the present invention.

#### EXPERIMENTAL EXAMPLE 4

The following experiment was carried out to identify whether the cancer vaccine according to the  
15 present invention can delete pre-existing tumor cells or not.

After dividing C57BL/6 mice into three groups, groups 1 and 2 were injected intraperitoneally with  $1 \times 10^4$  cells and  $1 \times 10^5$  cells of B 16 melanoma, respectively, and group 3 was injected intraperitoneally with  $1 \times 10^5$  cells of B 16 melanoma and then treated with the active cancer vaccine after allowing 48 hours for tumor cells to be completely engrafted.

20 Viability of mice was investigated according to time, and the result is given in FIG. 5. As shown in FIG. 5, viability of the group 3 treated with the active cancer vaccine was remarkably improved in comparison with groups 1 and 2. This result demonstrates that the active cancer vaccine is effective in destroying the previously formed tumor cells, as well as inhibiting incidence of tumors.



## EXPERIMENTAL EXAMPLE 5

To identify whether the cancer vaccine according to the present invention shows antitumor activity through mediation by NKT cells and CD1d, CD1d-deleted knock-out mice (CD1d<sup>-/-</sup>) and normal young mice (littermate, CD1D<sup>+/-</sup>), born of the same mother as the knock-out mice, were treated with the active vaccine.

Group 1 was composed of CD1d-deleted mice (CD1d<sup>-/-</sup>) treated with the active vaccine, group 2 was composed of normal mice (CD1D<sup>+/-</sup>) treated with the active vaccine, and group 3 was composed of treated with the inactive vaccine. 2 weeks after the treatment with the active or inactive vaccine, mice were injected with  $1 \times 10^5$  tumor cells. Viability of mice was evaluated, and the results are given in FIG. 6.

As shown in FIG. 6, the group 1 (CD1d<sup>-/-</sup>) with deletion of NKT cells was not improved in viability even though being treated with the active vaccine. Also, the group 3 (CD1D<sup>+/-</sup>) treated with the inactive vaccine, which was used as a control, did not show increased viability. Improved viability was observed in the group 2 having NKT cells, treated with the active vaccine. This result indicates that the immune response against tumor cells, induced by the active vaccine, is dependent on action of NKT cells and CD1d.

## EXPERIMENTAL EXAMPLE 6

As demonstrated in the Experimental Example 5, the immune response against tumor cells, induced by the active cancer vaccine according to the present invention, is dependent on NKT cells. In this experiment, it was investigated whether the immune response is induced by direct action of NKT cells or not.

Mice were treated with alpha-GalCer, which is one ingredient of the active cancer vaccine

according to the present invention. After 16 hours, the liver was excised from mice, and change in numbers of NKT cells was evaluated. The results are given in FIG. 7 in which the upper and lower figures at the left side are results when being treated with a solvent and used as controls, and the right upper and lower figures are results when being treated with alpha-GalCer. Also, in FIG. 7, NK1.1 and CD1-Tetramer on the y-axis, and TCRab and CD5 on the x-axis represent antigens immunostained in the immune cells.

As shown in FIG. 7, when being treated with alpha-GalCer, NKT cells were activated rapidly and then entered the programmed cell death process, resulting in rapid reduction of NKT cells, indicating that NKT cells themselves do not directly attack tumor cells injected after 2 weeks.

#### EXPERIMENTAL EXAMPLE 7

The following experiment was performed to identify whether the antitumor activity of the active cancer vaccine results from acquired immune response or not.

C57BL/6 female mice were immunized through injection of spleen cells from C57BL/6 male mice, in which the immune response was induced in a specific manner for male spleen cells (male mice-specific antigens are called H-Y antigens), along with treatment with alpha-GalCer capable of stimulating NKT cells. A control was treated with only a solvent (vehicle).

After 2 weeks, spleen cells were isolated from female mice. A cytotoxicity test was then carried out using the isolated spleen cells as effector cells to determine whether the spleen cells from female mice have cytotoxic activity against male mice-derived cells in vitro exposed to radiation for 5 days. The results are given in FIG. 8 in which the ratio of the effector cells (female mice-derived spleen cells, E) and the target cells (male mice-derived cells, T) is shown on the x-axis, and target-specific cytotoxic activity of spleen cells from female mice is shown on the y-axis.

As shown in FIG. 8, the effector cells from female mice treated with alpha-GalCer were found to

have about 100 times higher cytotoxic activity than those from the control. For example, the E:T ratio showing 40 % target-specific cytotoxic activity was 100 in the control, while being only about 1 in the case of being treated with alpha-GalCer, indicating that in the case of being treated with alpha-GalCer, one effector cell has cytotoxic activity equivalent to 100 effector cells from the control. On the other hand, when using target cells from female mice, no cytotoxic activity was observed.

This result indicates that the cytotoxic activity against specific antigens can be increased remarkably when the specific antigens are injected together with activation of NKT cells. Moreover, when the antigens are tumor cells, the cytotoxic activity against tumor cells can also be remarkably enhanced, thus illuminating the mechanism of the active cancer vaccine destroying or killing tumor cells.

#### EXPERIMENTAL EXAMPLE 8

With the same aim as in Experimental Example 7, this experiment was performed using tumor cells.

CD1d<sup>+</sup> mice were treated with the active cancer vaccine or the inactive cancer vaccine (groups 1 and 2, respectively), and CD1d<sup>-</sup> mice (group 3) were treated with the active cancer vaccine. After 2 weeks, spleen cells (effector cells) were isolated from each group, and in vitro activated with tumor cell extract for 5 days. Cytotoxic activity of the effector cells against live tumor cells (target cells) was evaluated, and the results are given in FIG. 9.

As shown in FIG. 9, group 1 treated with the active cancer vaccine, in which NKT cells normally exist, showed remarkably increased cytotoxic activity against B 16 melanoma, and group 2 treated with the inactive cancer vaccine showed several tens times lower cytotoxic activity than that of group 1. On the other hand, when being treated with the active cancer vaccine, group 3

(NKT cell-deleted CD1d<sup>-/-</sup> mice), which was used as a control, was found to have weak cytotoxic activity.

These results indicate that the cytotoxic activity of the active cancer vaccine is obtained from increased cytotoxic reaction mediated by the activated NKT cells in a manner dependent on  
5 CD1d. Therefore, the active cancer vaccine according to the present invention can be used for development of effective vaccines for cancer as well as other diseases.

NKT cells can be activated through use of antibodies capable of selectively recognizing and activating NKT cells, and antibodies capable of stimulating T cell receptor (TCR) on NKT cells,  
10 as well as alpha-GalCer.

The antibodies capable of stimulating NKT cells are well known in the art. For example, anti-NK1.1 antibody interacting with NK1.1, which is a surface antigen of NKT cells, is theoretically considered to have sufficient ability to activate NKT cells. In addition, antibodies capable of recognizing NKT cell-specific T cell receptor (Va14Ja281) can be used.

15 Taken together, when animals or humans are immunized with the cancer vaccine according to the present invention using the necrotic tumor cells as an antigen, simultaneously with activation of NKT cells, the immunological tolerance, leading to deletion of the immune cells capable of recognizing tumor cells, can be inhibited, the immune system can be increasingly activated by various signals, and memory T cells against tumor cells can be finally formed, thus making it  
20 possible to vaccinate against and treat cancer.

#### INDUSTRIAL APPLICABILITY

As described hereinbefore, when being immunized with the pharmaceutical preparation for  
25 preventing and treating cancer according to the present invention, the immune system can

recognize tumor cells as cancer-specific antigens, not “self”, and increase production of the co-stimulatory molecules required for maximal T cell activation, in which NKT cells can be activated by the material capable of stimulating NKT cells, contained in the pharmaceutical preparation, thus inducing the immune response against tumor cells. Therefore, the pharmaceutical preparation is  
5 very useful for prevention and therapy of cancer, with a potential use as a cancer vaccine.

## CLAIMS

1. A pharmaceutical preparation for preventing and treating cancer, comprising a biologically effective amount of killed tumor cells, which act as antigens, and a biologically effective amount of  
5 a material to increase expression of a co-stimulating molecule required for maximal T cell activation, as effective ingredients.
2. The pharmaceutical composition as set forth in claim 1, wherein the killed tumor cells are necrotic cells.
- 10 3. The pharmaceutical composition as set forth in claim 1 or 2, wherein the co-stimulatory molecule is B7-1 or B7-2.
4. The pharmaceutical composition as set forth in claim 3, wherein the material to increase  
15 expression of the co-stimulatory B7-1 or B7-2 molecule is selected from materials stimulating NKT cells, which activate antigen presenting cells (APC) expressing the B7-1 or B7-2 molecule.
5. The pharmaceutical composition as set forth in claim 4, wherein the material to activate NKT cells is glycolipid or lipid acting as a ligand for CD1d molecule on antigen presenting cells that  
20 present antigen to NKT cells.
6. The pharmaceutical composition as set forth in claim 5, wherein the glycolipid is alpha-galactosylceramide (alpha-GalCer).
- 25 7. The pharmaceutical composition as set forth in claim 4, wherein the material to activate NKT

cells is an antibody selectively recognizing and activating NKT cells.

8. The pharmaceutical composition as set forth in claim 4, wherein the material to activate NKT cells is selected from materials stimulating T cell receptor (TCR) on NKT cells and thus activating

5 NKT cells.

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FIG 1

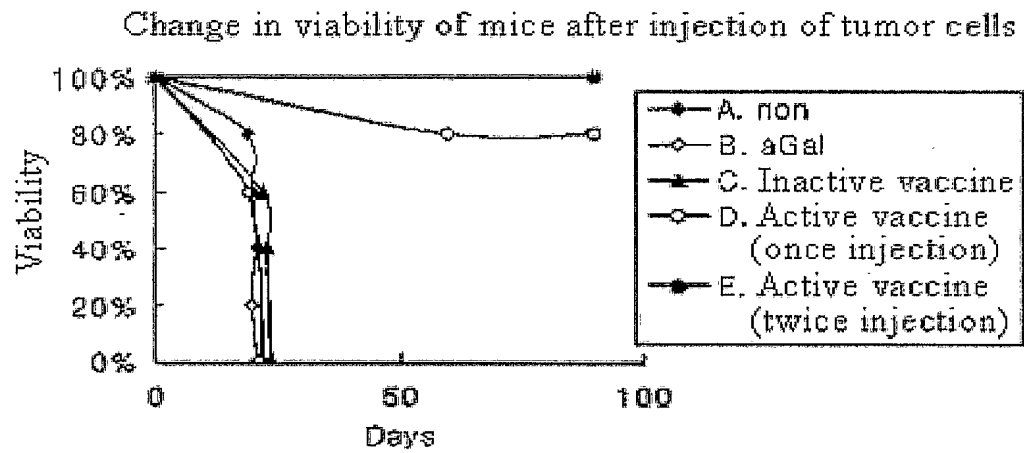
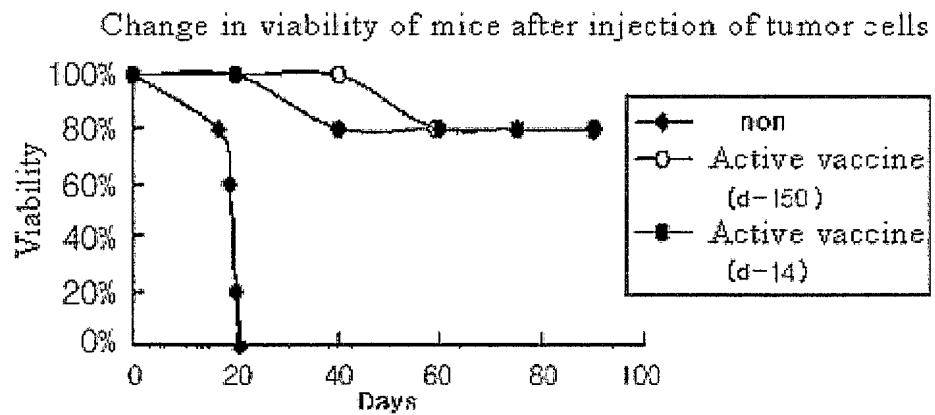


FIG 2





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FIG 3

DC activation after NKT cell  
Stimulation by  $\alpha$ GalCer injection

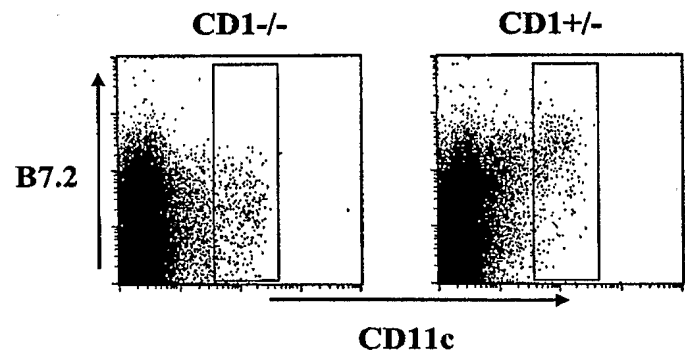
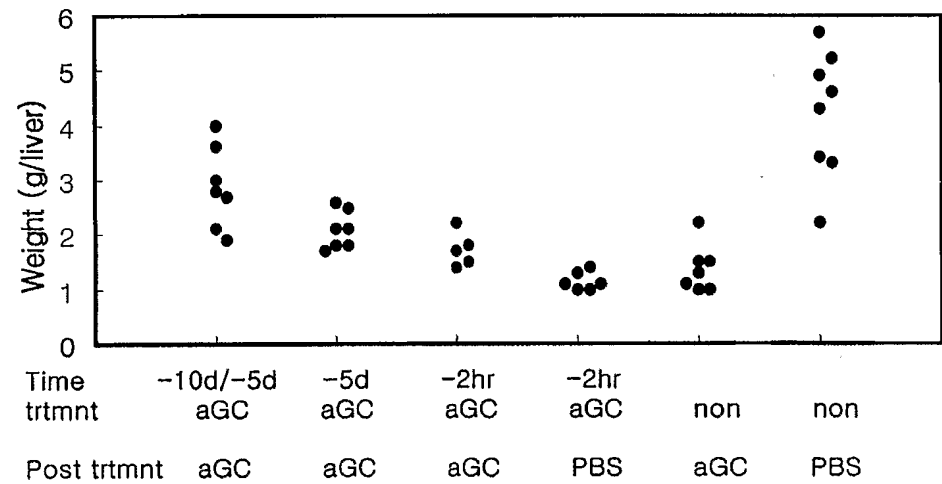


FIG 4

Pre-treatment effect of  $\alpha$ GalCer



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FIG 5

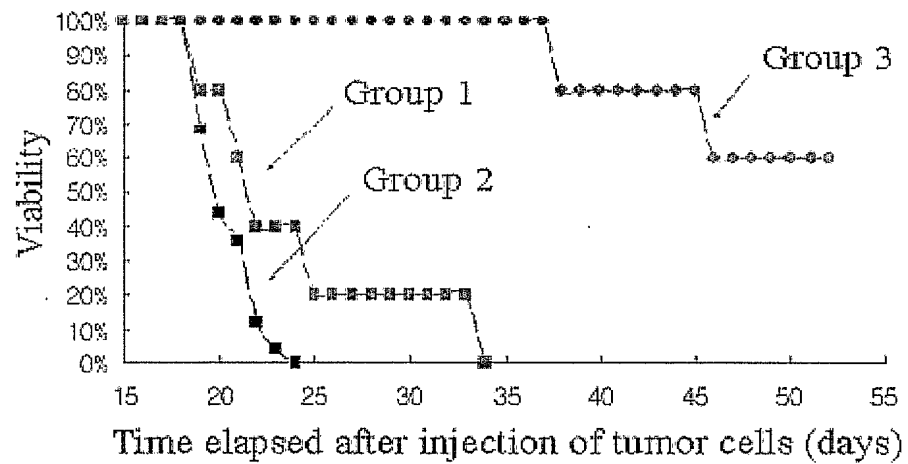
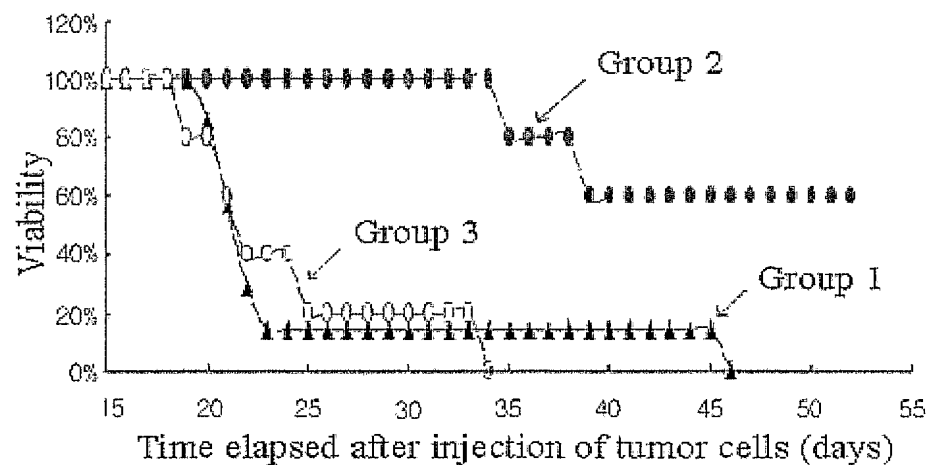


FIG 6



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FIG 7

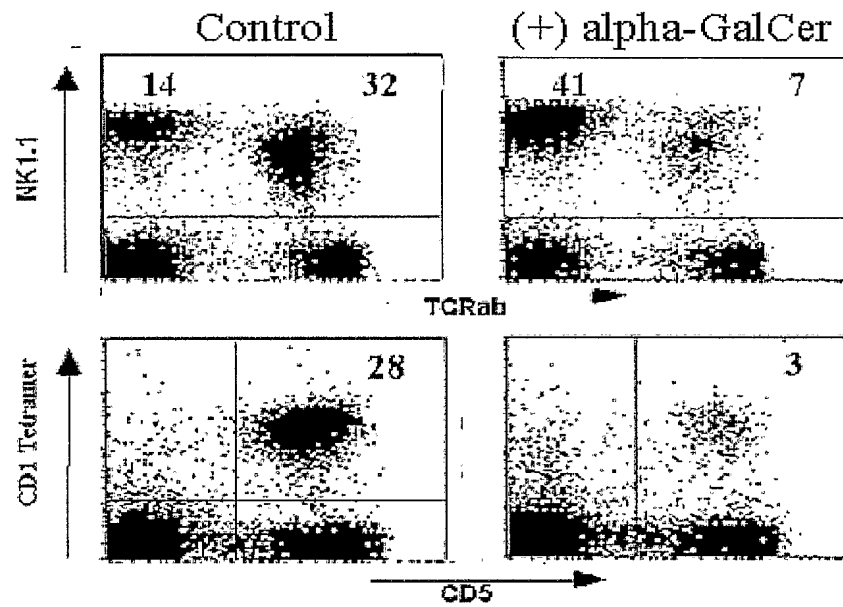
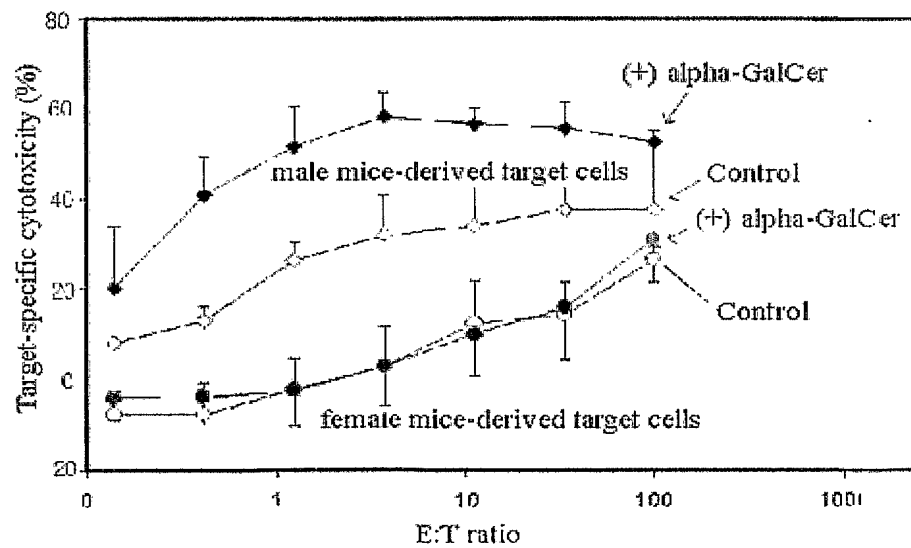
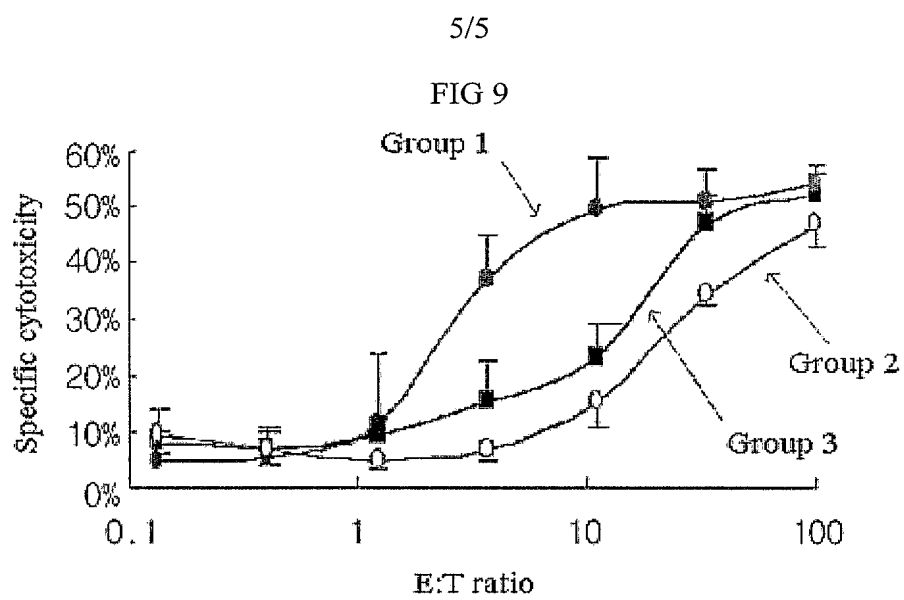


FIG 8





# INTERNATIONAL SEARCH REPORT

national application No.  
PCT/KR02/01585

## A. CLASSIFICATION OF SUBJECT MATTER

**IPC7 A61K 35/12**

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 07 A61K 35/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, Pubmed

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Soichiro Ishihara, et al., "alpha-Glycosylceramides Enhance the Antitumor Cytotoxicity of Hepatic Lymphocytes Obtained from Cancer Patients by Activating CD3-CD56+ NK Cells In Vitro" In Journal of Immunology, Vol.165, No.3, 2000, p.1659-64 See the whole document	1-8
Y	Liu Yang, "T-cell costimulation and vaccine" In Designer Vaccines, 1998, p.23-41 See the whole document	1-8
A	Thomas Felzmann, et al., "Functional maturation of dendritic cells by exposure to CD40L transgenic tumor cells, fibroblasts or keratinocytes" In Cancer Letters Vol.168, No.2, 2001, p.145-54 See the whole document	1-8
A	WO 01/54176 A2 (SIDNEY KIMMEL CANCER CENTER) 02 AUGUST 2001 See the whole document	1-8
A	KIM KD, et al., "Protective antitumor activity through dendritic cell immunization is mediated by NK cell as well as CTL activation" In Archives Pharmaceutical Research Vol.22, No.4, 1999, p.340-7 See the whole document	1-8



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents:

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Date of the actual completion of the international search

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR02/01585

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 01/54716 A2	02.08.01	US 2002006413 AA AU 200131204 A5	17.01.02 07.08.01